Application of autologous cryopreserved bone marrow mesenchymal stem cells for periodontal regeneration in dogs

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ABSTRACT

Background: Stem cell based therapies have shown great promise in regenerative medicine and continue to generate wide interest in future clinical applications. However, the issue of storage and preservation of stem cells, for future clinical applications, still requires extensive investigation.

Objective: The purpose of this study was to evaluate the effect of cryopreservation on the regenerative capacity of bone marrow stem cells in periodontal defects in dogs.

Materials and methods: Bone marrow mesenchymal stem cells (BMSCs) were obtained from five Beagle dogs. After cryopreservation for one month the measure of cell viability, surface adherence ability, alkaline phosphatase activity and mineralized nodule formation was assessed. 26 periodontal fenestration defects (5mm×5mm) were created at a location of 5mm apical to the cementoenamel junction in experiment teeth. Cryopreserved BMSCs were transplanted into the defects using a collagen scaffold carrier. Freshly isolated BMSCs and collagen scaffold alone were used as controls. All animals were sacrificed eight weeks after surgery and specimens were evaluated by histomorphometry.

Results: Cryopreservation had no discernible negative effect on BMSCs growth and differentiation *in vitro*. Both freshly isolated and cryopreserved BMSCs transplantations induced significantly better periodontal regeneration with newly formed cementum, alveolar bone and periodontal ligament compared with the application of collagen scaffold alone.

Conclusion: Cryopreserved BMSCs showed no altered regenerative capacity

compared with freshly isolated BMSCs in the application of periodontal regeneration.

INTRODUCTION

Periodontal regeneration is a persistent challenge for periodontist in the treatment of chronic periodontitis [Kawaguchi et al., 2004]. Progress in tissue engineering - which consists of three basic elements: scaffold, cell, and growth factors - has opened up the field of regeneration of various tissues and organs. Mesenchymal stem cells (MSCs) are multipotential cells and can be induced, *in vitro* and *in vivo*, to differentiate into various functional types of mesodermal tissues, including bone, cartilage, tendon, fat and nerves [Pittenger et al., 1999]. MSCs have therefore been proposed as a potential type of seeding cells in periodontal tissue engineering. Indeed, BMSCs have shown the capacities to differentiate into cementoblast, osteoblast, periodontal fibroblast, and secret the extracellular matrix of periodontal ligament, cementum and alveolar bone [Kawaguchi et al., 2004; Hasegawa et al., 2006]..

It is known that the number of BMSCs decline with ageing and the BMSC number that can be culture-expanded from fresh bone marrow aspirate, over a short period of time, is limited and that the prolifereative capacity of BMSCs decline during repeat passages over an extended culture period. Therefore, in order to avoid the repeated harvestings for each treatment of a patient, cryopreservation of therapeutic cells is necessary. This is especially significant in the case of patients with a limited supply of mesenchymal cells [Kotobuki et al., 2005]. However, the freezing and thawing process is potentially detrimental to cell viability. During conventional freezing, water precipitates as ice and often leads to tissue damage, which acts on cytoplasmic structures, even influencing cytoskeleton or genome-related structures [Martin et al.,

2004]. Interestingly, Bruder and colleagues [Bruder et al., 1997] compared frozen and unfrozen BMSCs populations in parallel, for as many as 15 passages, and found that freezing had no discernible influence on *in vitro* cell biological characteristics of the cells. BMSCs obtained from fresh human bone marrow aspirates can be cryopreserved without compromise to their osteogenic potential [Kotobuki et al., 2005] and are capable of differentiating into adipocytes, chondrocytes, osteoblast , and neurocytes *in vitro* [Xiang et al., 2007]. Cryopreserved BMSCs also retain a marked *in vivo* osteogenic capacity [Yoshikawa et al., 1996] . To our knowledge, no previous studies have addressed the influence of cryopreservation on the capability of BMSCs to regenerate periodontal tissue *in vivo*, although some researchers [Kawaguchi et al., 2004; Hasegawa et al., 2006; Weng et al., 2006] have demonstrated reasonable periodontal regeneration using BMSCs in periodontal tissue engineering. Therefore, the aim of our study was to investigate the biological features of resuscitated BMSCs from cryopreservation and their regenerative capacity in periodontal tissue regeneration.

MATERIALS AND METHODS

Isolation and culture of MSCs

This study was performed under protocols approved by the University Committee on Use and Care of Animals of the Fujian Medical University. Five healthy adult female Beagle dogs, weighing between 12-18kg, were used in the experiment. Two

milliliters of bone marrow was aspirated from the femur of each dog into a 50ml tube, containing 30ml Dulbcco's Modifed Eagle Medium (Gibco,US) and heparin (100U/mL). The mixture was centrifuged at 400g without acceleration or brake for 35 min at 20ºC on Ficoll gradient media and the top layer of fat containing plasma was discarded. Cells located at the interface between the bone marrow sample and gradient media were collected, washed and recentrifuged at the same speed. The residue transferred into a 10-cm plate with complete DMEM with 10% FBS (Jb-bio, China) and 1% penicillin-streptomycin and cultured in a humidified atmosphere of 5% CO_2 at 37ºC. The medium was changed every 2-3 days until the outgrown cells were subconfluent, after which nonadherent cells were washed off with PBS and the adherent cells detached with trypsin-EDTA (Gibco, USA) and subcultured in 10-cm dish at a 1:2 split ratio.

Cryopreservation and resuscitation

At passage four, 8 separate BMSC cell lines were trypsinized and divided into two aliquots each. Half of the samples (8) were prepared for cryopreservation at a density of 1×10^6 cells/ml, suspended in DMEM containing 10% FBS with 10% dimethyl sulfoxide (DMSO) (Shanghai Chemical, China) as a cryoprotectant. The cells were transferred to cryovials and frozen in a three step sequence modified from Kotobuki and Hirose et al[Kotobuki et al., 2005]. Briefly the cells were stored at 4℃ for 1hr, -20℃ for 2hrs, -80℃ for 10.5hrs, and finally, stored in liquid nitrogen (-196℃) for long term storage. Thawing out of the BMSCs was done at 37℃ in order to minimize

cellular damage.

In vitro biological characteristics

To measure *in vitro* biological characteristics, BMSCs cryopreserved for one month and noncryopreserved cells were sub-cultured to passage 7. Cell viability was measured by trypan blue exclusion. Fresh and frozen-thawed BMSCs were incubated in triplicates in a 0.4% trypan blue-dye solution (Gibco, USA). Trypan blue–positive cells were determined by counting at least 200 cells per sample in a hemocytometer. Cell viability percentage was calculated by the ratio of trypan blue–impermeable cells relative to the total cell count (trypan blue–impermeable cell number/total cell number).

The number of adherent cells between cryopreserved and noncryopreserved cultures of BMSCs was assessed by seeding 5×10^4cells/ well into 24 well-plates. At 4, 8, 12, and 24hrs non-adherent cell were removed by washing the wells four times with PBS. The remaining adherent cells were trypinized and counted with a hemocytometer and the percentage of adherent cell used for the comparison assessment. Each assay was performed in triplicates.

In vitro osteogenic differentiation

Cryopreserved and noncryopreserved cells were seeded into 96 well-plate at 1×10^5 cells/ml in 200μl mineralization culture media containing 50μM ascorbic acid, 10mM β-glycerol phosphate and 100nM dexamethasone in DMEM with 10% FBS, and alkaline

phosphatase (ALP) activity determined on days 5, 10 and 15. Briefly, the cells were washed in PBS and scraped in 10 mM Tris-HCl buffer (pH = 7.6) containing 10mM $MgCl_2$ and 0.1% Triton X-100 (Biosino, china). 100μl disodium nitrophenyl phosphate at 3mmol/L was added, and then incubated at 37°C for 30 min. After the mixture was cooled to 4℃ for 12h, 50μl 0.02 M NaOH was added, and absorption of *p*-nitrophenol was measured with a spectrophotometer at 405 nm against a reagent blank. ALP activity was expressed as the formation of *p*-nitrophenol in nM per minute.

Mineralized nodule formation

Cultures of BMSCs were initiated as previously described[Wang et al., 2006]. Briefly, BMSCs were cultured in two-well-plates at a density of 2×10^7 cells/well. After 48 hrs, non-adherent cells were removed by rinsing with PBS and cultures were maintained in mineralization culture media containing 50μM ascorbic acid, 10mM β-glycerol phosphate and 100nM dexamethasone in DMEM with 10% FBS for a further 21days, with twice weekly media changes. Cultures were fixed in a 1:1:1.5 solution of 10% formalin/methanol/water for 3 hrs and von Kossa stained for the assessment of mineralization. This method stains mineralized matrix in black and non-mineralized matrix in dark yellow. The number of mineralized nodules was determined macroscopically with a magnifying glass over transmitted light.

BMSC seeding on collagen scaffolds

Prior to *in vivo* transplantation, the collagen membranes (BMT Bio, China) were trimmed to a size of 5mm×5mm and sterilized with thylene oxide gas, then soaked in DMEM to facilitate cell-scaffold combination. Twenty-four hours prior to implantation into the periodontal defect, 20 μl of 5×10^6 / ml noncryopreserved or resuscitated BMSCs were seeded onto the collagen membrane scaffold and incubated for 24 hours to enhance cell-scaffold interaction.

A scanning electron microscopy was used to observe the extent of cell attachment on the collagen scaffold. 24 hours after seeding, samples were fixed in 3% (v/v) glutaraldehyde (Sigma) and 1% (wt/vol) osmium tetraoxide (Sigma), dehydrated in a graded series of ethanol (Sigma), dried and sputter coated with gold-palladium alloy. Each scaffold sample was imaged at 5 kV on a JEOL JSM-6330F field emission Scanning electron microscopy (Philips XL30, NLD). .

Animal experiment

Complete scaling of experimental teeth was utilized to acquire satisfactory periodontal status before autologous transplantation of cell-scaffold complex. From five experimental animals, 26 teeth were selected for the study, which included 6 bilateral upper canines, 10 lower canines, and 10 lower first molars. Eight teeth were used for the control group, which contained 2 bilateral upper canines, 3 lower canines and 3 lower first molars. Nine teeth were used for the noncryopreserved cell-scaffold group, which contained 2 bilateral upper canines, 4 lower canines and 3 lower first molars. Nine teeth were used for the cryopreserved cell-scaffold group, which

contained 2 bilateral upper canines, 3 lower canines and 4 lower first molars. Scaffolds without MSCs were implanted into the experimental defect in the control group. A partial block design approach was used to allocate teeth to treatments such that each dog received treatments covering all three scaffold groups and the treatment location was equally distributed in the treatment groups.

All surgical procedure were performed under both systemic (ketamine 10mg/kg) and local anesthesia (2% lidocaine containing 1:80,000 epinephrine), followed by intramuscular injection of atropine sulfate (50mg/mL). Periodontal fenestration defects were modified from the model used by Nakahara and Nakamura et al [Nakahara et al., 2004]. Following the elevation of buccal mucoperiosteal flaps, the fenestration defects (5mm×5mm) were established 5mm apical to the cemento-enamel junction in the experimental teeth. The alveolar bone was removed by a dental bur at high speed under saline irrigation. The exposed buccal root surfaces were carefully denuded of its periodontal ligament, overlying cementum, and superficial dentin with manual instruments (Figure 1A). The 5mm×5mm collagen membrane or collagen-cell complex was placed into the surgical defects with the cell-seeded side contacting root surface, using gentle pressure to place the implants in close approximation with the root surface. Then the defects were completely covered with an e-polytetrafluoroethylene (e-PTFE) membrane that was trimmed and fixed in place by hammering titanium bone tacks (0.5 mm in diameter) (Zhongbang, China) in the normal bone areas around the defects without damaging the roots (Figure 1 B). Mucoperiosteal flaps were subsequently repositioned and sutured. After the surgery

animals were intramuscularly applied penicillin (800,000 units) twice a day for 4 days and were on soft diet for a week. All the dogs were sacrificed 8 weeks after surgery. The samples were dissected and fixed in 10% buffered formalin, decalcified in formic acid and sodium formate, and embedded in paraffin. Serial sections (5μm) were cut in the buccal-lingual plan throughout the mesial-distal extension of the tooth. The sections were stained with hematoxylin-eosin (H&E) and Masson trichrome for hisotological and histometric evaluations using a light microscope. In each sample 10 slices were cut and stained for the morphometric analysis of tissue healing.

Histological and histometric evaluation

The histological specimens were analyzed using a light microscope fitted with a camera and the regeneration of periodontal tissues was measured using analytic software (Olysia Bioreport 3.2, Japan). A single masked, calibrated examiner examined all of the slides with demonstrated calibration intra examiner error under 5%. The measured items were defined as follows (Figure. 1C):

1. Total defect height (TH): The length of fenestration defect between the two notches.

2. Continuous regenerated cementum (CC): The length between the defect margin and the apical or coronal extension of the newly regenerated cementum continuous with the existing cementum.

3. Isolated regenerated cementum (IC): The length of the isolated newly formed cementum not continuous with the old cementum.

4. Continuous regenerated bone (C**B**): The length between the defect margin and the apical or coronal extension of the newly regenerated bone continuous with the existing alveolar bone.

5. Isolated bone regeneration(IC): The length of the isolated newly formed bone not continuous with the old alveolar bone.

6. New periodontal ligament (NP): The length between the defect margin and the apical or coronal extension of the newly regenerated periodontal ligament.

The new cementum (NC), new PDL (NP) and new alveolar bone (NB) formed along the denuded root surface on each specimen were added, and then divided by the total defect height to acquire the percentage of the regeneration for further statistical analysis.

Statistics

All data were statistically analyzed with the Mann-Whitney U test. Significance level was set at $p \leq 0.05$.

RESULTS

Our method of isolation and ex vivo expansion of BMSCs was proven to be successful in BMSC culture. Spindle-like cells adhered to the plastic plate after 72 hr incubation. Colonies formed at days 7-9 and a cell monolayer was apparent after 2 weeks (Fig 2a). Cryopreserved BMSCs regained a typical spindle-like shape after the second passage of thawed BMSCs from liquid nitrogen.

The viability rate between unfrozen and frozen BMSCs was 94.1±1.67% and 90.1±2.57% respectively, with no significant difference between two groups. Cell attachment studies revealed that the resuscitated BMSCs had decreased adhesion capacity compared to unfrozen cells indicating that the cryopreservation process may have some effect on cell surface structure. However, after cultured in normal culture media for several passages (passage 7), cell attachment capacity recovered and no difference was detected compared to unfrozen BMSCs (data not shown). Comparison of *in vitro* differentiation capacity between frozen and unfrozen BMSCs indicated that ALP expression changed in a time-dependent manner in both types of BMSCs (Fig 3), suggesting that frozen BMSCs had comparable capacity for osteogenic differentiation. Similarly, a roughly equal number of mineralization nodules was observed in both noncryopreserved cells (Fig 2b) and cryopreserved cells by von Kossa staining (Fig 2c).

Collagen scaffolds used in this study showed a smooth surface structure and both frozen and unfrozen BMSCs grew well on these surfaces. Cell migration into porous areas was observed 24 hours after incubation (Fig 2d). There was no detectable difference in cell growth on collagen scaffolds between cryopreserved and noncryopreserved BMSCs.

All the animals recovered well after periodontal surgery with the exception of one site of the low first molar which suffered an infection after the implantation of cryopreserved BMSC. This specimen was discarded. During the retrieval of samples from the animals, the e-PTFE membrane remained fixed on the fenestration

defects without collapsing after elevation of the mucoperiosteal flaps. However, during the decalcification and tissue procession some e-PTFE membrane separated from the tissue, which resulted in no membrane appearance in some tissue sections.

In both the cryopreserved and noncryopreserved BMSC-scaffold transplantation groups, newly formed periodontal tissue was observed (Fig 4). Nearby formed cementoid structure and periodontal ligament was clearly visible in the microscope (Fig 4A-H). Islands of newly regenerated alveolar bone were seen. These osteoid showed lower degree of calcification and greater cellular component (Fig 4C and 4G). Spindle-shaped cementoblast-like cells lined the newly formed cementum and lay in vacuoles in cementum (Fig 4C&D and 4G&H). Newly formed periodontal ligament was formed between the new alveolar bone and cementum with Sharpey's fibers extending into the newly formed cementum and bone (Fig 4C&D and 4G&H).

In the control group (collagen scaffold alone) very few regenerated alveolar bone and cementum were observed (Fig 4I-L). Recovered periodontal ligament fibers ran parallel to the root surface but few cementoblasts were observed on the root surface. No difference was observed in tissue healing between the different defect locations in upper canines, lower canines and first lower molar.

Implanted collagen sponge scaffolds had degraded and been replaced by host tissues in both the BMSCs-scaffold groups whereas, parts of collagen scaffold remained in the transplantation sites in the application of collagen membrane alone. Moreover, small lacunae of root resorption were seen on some of the root surfaces of the control group. The result of histometric analysis showed that there was no significant

difference in the percentage of alveolar bone regeneration between the cryopreserved cell group (83.7±10.6) and the noncryopreserved cell group (85.0±6.8). Cementum and PDL were fully regenerated in both cryopreserved and fresh isolated BMSC groups. However in the control group significantly less alveolar bone regeneration (24.7±9.2), PDL regeneration (24.8±5.4), and cementum regeneration (21±4.8) were observed compared with the both cell delivery groups (Table 1).

DISCUSSION

Tissue engineering, using patient-derived cells, may potentially play a major role in the future of periodontal treatment. Autologous BMSCs have the advantage of being non-immunogenic, highly proliferative, easy to harvest, and having greater ability to differentiate into various types of cells, including periodontal tissue, and could therefore be widely used in future periodontal regeneration. If mesenchymal stromal cells collected from young patients can be cryopreserved and still maintain high proliferation, differentiation, and *in vivo* tissue forming capabilities, BMSCs would be the best potential source of cells for future applications in the treatment of age-related disorders such as periodontitis. However, freezing may cause physical disruption to cell structures leading to biochemical changes. The cooling rate and concentration of cryoprotectants applied to the cells are two of the main factors governing the survival of frozen cells [Limaye, 1997]. A slow and controlled cooling rate in cryopreservation is conventionally applied by commercially available cell-freezing systems. However, this procedure both requires expensive equipment and is relatively time consuming. The three-step cryopreservation method used in this study, including the use of

DMSO as a cryoprotectant, is an osmotic process and works by reducing the electrolyte concentration in the residual unfrozen solution in and around a cell at a given temperature. A previous study reported that cellular damage may occur in the process of freezing and thawing cells [Mazur, 1970]. In our study, the attachment properties of BMSCs, freshly resuscitated from cryopreservation, decreased indicating a potentially detrimental effect on the cells by the cryopreservation process. However, cell attachment ability could appeared to recover after culturing a further two passages, demonstrated by the *in vitro* cell viability and cell attachment assays. Interestingly, the freezing and thawing process showed no negative effect on *in vitro* osteogenic differentiation. Similar studies have also demonstrated that BMSCs stored cryogenically long-term for 3 years were still able to maintain high osteogenic potential *in vitro* [Kotobuki et al., 2005] and *in vivo* [Shimakura et al., 2003].

The concept of cell delivery therapies in periodontal regeneration has been tested using periodontal ligament cells to generate cementoid structures on the root surface [Karring et al., 1993] and BMSCs to gain the characteristics of periodontal tissues [Hasegawa et al., 2006]. It has also been suggested that the local microenvironment and surrounding tissues may provide the nutrients, growth factors and extracellular matrices necessary to support differentiation of the transplanted BMSCs [Krause et al., 2001]. Our study has demonstrated that upon transplantation, cryopreserved BMSCs can support the regeneration of cementum, periodontal ligament and alveolar bone tissues in a canine periodontal defect model. These results are consistent with previous reports concerning transplanted BMSCs [Kawaguchi et al., 2004; Hasegawa

et al., 2006]. The exact mechanism by which BMSCs differentiate into periodontal cells is still unknown. It is expected that transplantated multipotential BMSCs may induce the early differentiation signals in the defect microenvironment and play a critical role in cellular interaction with host mesenchymal stem cells to generate periodontal tissues. No significant difference was found in the regenerative abilities between the frozen and unfrozen BMSCs, suggesting the cryopreservation had no discernible negative effects on the *in vivo* ability of BMSCs to regenerate periodontal tissue.

In this study we utilized collagen as the biomaterial scaffold to hold and support BMSCs during transplantation. The collagen matrix gradually degraded and was substituted by the newly regenerated tissues in the periodontal defects. It was noted that less collagen matrix residue was found in either the cryopreserved or non-cryoprerserved BMSCs-scaffold groups, compared with the collagen scaffold alone. It is not known whether collagen residue has some effect on periodontal wound healing or not. In this study when the defect was only filled with collagen alone, no newly formed cementum was observed and limited new bone formation could be detected. Compared with the other studies tissue response was relatively poorer in the control group. The reason is not clear, but may be related with the degradation of filling materials. Future study will be needed to determine the degradation of collagen scaffold is due to the activation of phagocytosis by the monocytes in the defect area by transplanted BMSCs or/and an increase in the level of matrix metalloproteinases (MMPs) in the transplantation sites secreted by transplanted BMSCs and

differentiated BMSCs. It will be interesting to know how this process effects on tissue regeneration.

In this study we observed certain amount of periodontal regeneration tissues in the control group although less organized in the structure than the cell-carrier groups. It has been reported that nearly 50% to 70% spontaneous regeneration can be expected in acute defect models in primate [Caton et al., 1994]. We found 25% spontaneous periodontal regeneration in the dog model. It is conceivable that spontaneous periodontal regeneration in the control group is derived from stem cells from local periodontal ligament and alveolar bone marrow as well as cells from normal cementum, which has been shown with a capacity of differentiating and forming a cementum-like tissue when transplanted into immune compromised mice [Grzesik et al., 2000].

The periodontal defect model used in this study has been used by many other investigators [Nakahara et al., 2004; Zhao et al., 2004] and proven to be a valuable model for studying periodontal tissue healing. It has been used as a reasonably simple screening model to examine wound healing kinetics. However, periodontal defect resulting from periodontal diseases are far more complicated than the animal model used in this study. Periodontal pathogens could play an important role in wound healing by compromising the regeneration process. Case studies, using MSCs in human periodontal treatment, have demonstrated promising clinical outcomes in periodontitis-related periodontal defects [Kawaguchi et al., 2004; Yamada et al., 2004; Kawaguchi et al., 2005]. However, more studies into BMSC delivery therapies are

required in order to fully understand the cell interactions taking place during tissue formation, as well as optimizing cell delivery strategies for tissue engineering of complicated periodontal defects.

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LEGENDS

Figure 1 Illustration of the fenestration defect model and diagram of histometric assessments

Figure 1A: Representative picture of the entire fenestration defects. Figure 1B: Covering the defect by ePTFE membrane using bone tacks. Figure 1C: Diagram of histometric assessments. OB: existing alveolar bone; OC: existing cementum; CB: continuous regenerated bone; IB: isolated regenerated bone; CC: continuous regenerated cementum; IC: isolated regenerated cementum; D: dentin

Figure 2 Cell morphology and mineralization capacity of cryopreserved and noncryopreserve canine bone marrow mesenchymal stem cells

Primary BMSCs showed spindle-shaped appearance under microscope on the 4th day of harvest (A). Both the cryopreserved (B) and noncryopreserved (C) BMSCs showed mineralization ability *in vitro* by von Kossa staining (×100). Scanning electron microscopy examination of the collagen membrane showed the porous structure of collagen scaffold (D). BMSCs grew and proliferated well after seeding onto collagen scaffold (arrow head) (E).

Figure 3 Alkaline phosphatase activity at passage 7 of the cryopreserved and noncryopreserved BMSCs.

ALP activity increased in a time-dependent manner with significantly upregulation in day 10 and 15 in both cryopreserved and noncryopreserved BMSCs. No significant

difference was found on days 5 and 10 between the two types of BMSCs. However, ALP activity in the noncryopreserved BMSCs showed slightly higher than ceyopreserved BMSCs in day 15.

Figure 4 Periodontal regeneration in cryopreserved, fresh BMSCs-collagen scaffold groups and control group eight weeks after surgery

In cryopreserved BMSC transplantation, robust periodontal tissue regeneration was observed under H&E (A) and Masson trichome (B) staining. Border of the experimental periodontal defect showed the difference between the newly formed periodontium (white arrow head) and the existing periodontium (C) and (D). Thin layer of newly regenerated cementum almost completely covered the denuded root surface (A) and (B). Sharpey's fibers (white arrow) were detected linking the newly formed cementum with adjacent alveolar bone (C) and (D).

In fresh BMSC transplantation, e-PTEF membrane covered the fenestration defect (E and F) to prevent epithelium invasion. Newly formed alveolar bone can be seen in the defects (white arrow in G). Thin layer of cementum covered the root surface, and newly formed periodontal fibers connected the newly formed cementum and alveolar bone (G and H). Sharper's fibers could also be identified in the newly formed structures (G and H).

In the control group**,** newly formed alveolar bone was sparsely identified (I and J). Some minute areas of alveolar bone (white arrow head) and cementum (black arrow head) regeneration can be seen at the border of the defect (I and J). Layers of

periodontal fibers lay parallel to the root surface (K and L). Resorption vacuole was found on the dentin surface (white arrow in K and L).

NB: new bone, NC: new cementum, PDL: periodontal ligament, D: dentin, B: bone.

Table1. Percentage of periodontal tissue regeneration after surgery

Nearly complete PDL and cementoid structure regeneration was observed in the cryopreserved and noncryopreserved groups. Cell-scaffold group were significantly higher compared to the control group ($P<0.05$). Values were expressed as means ± standard deviation.

REFERENCES

Bruder, S.P., N. Jaiswal, S.E. Haynesworth (1997) Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. J Cell Biochem *64*: 278-294.

Caton, J., L. Mota, L. Gandini, B. Laskaris (1994) Non-human primate models for testing the efficacy and safety of periodontal regeneration procedures. J Periodontol *65*: 1143-1150.

Grzesik, W.J., H. Cheng, J.S. Oh, S.A. Kuznetsov, M.H. Mankani, K. Uzawa, P.G. Robey, M. Yamauchi (2000) Cementum-forming cells are phenotypically distinct from bone-forming cells. J Bone Miner Res *15*: 52-59.

Hasegawa, N., H. Kawaguchi, A. Hirachi, K. Takeda, N. Mizuno, M. Nishimura, C. Koike, K. Tsuji, H. Iba, Y. Kato, H. Kurihara (2006) Behavior of transplanted bone marrow-derived mesenchymal stem cells in periodontal defects. J Periodontol *77*: 1003-1007.

Karring, T., S. Nyman, J. Gottlow, L. Laurell (1993) Development of the biological concept of guided tissue regeneration--animal and human studies. Periodontol 2000 *1*: 26-35.

Kawaguchi, H., H. Hayashi, N. Mizuno, T. Fujita, N. Hasegawa, H. Shiba, S. Nakamura, T. Hino, H. Yoshino, H. Kurihara, H. Tanaka, A. Kimura, K. Tsuji, Y. Kato (2005) Cell transplantation for periodontal diseases. A novel periodontal tissue regenerative therapy using bone marrow mesenchymal stem cells. Clin Calcium *15*: 99-104.

Kawaguchi, H., A. Hirachi, N. Hasegawa, T. Iwata, H. Hamaguchi, H. Shiba, T. Takata, Y. Kato, H. Kurihara (2004) Enhancement of periodontal tissue regeneration by transplantation of bone marrow mesenchymal stem cells. J Periodontol *75*: 1281-1287.

Kotobuki, N., M. Hirose, H. Machida, Y. Katou, K. Muraki, Y. Takakura, H. Ohgushi (2005) Viability and osteogenic potential of cryopreserved human bone marrow-derived mesenchymal cells. Tissue Eng *11*: 663-673.

Krause, D.S., N.D. Theise, M.I. Collector, O. Henegariu, S. Hwang, R. Gardner, S. Neutzel, S.J. Sharkis (2001) Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell *105*: 369-377.

Limaye, L.S. (1997) Bone marrow cryopreservation: improved recovery due to bioantioxidant additives in the freezing solution. Stem Cells *15*: 353-358.

Martin, G., O. Sabido, P. Durand, R. Levy (2004) Cryopreservation induces an apoptosis-like mechanism in bull sperm. Biol Reprod *71*: 28-37.

Mazur, P. (1970) Cryobiology: the freezing of biological systems. Science *168*: 939-949.

Nakahara, T., T. Nakamura, E. Kobayashi, K. Kuremoto, T. Matsuno, Y. Tabata, K. Eto, Y. Shimizu (2004) In situ tissue engineering of periodontal tissues by seeding with periodontal ligament-derived cells. Tissue Eng *10*: 537-544.

Pittenger, M.F., A.M. Mackay, S.C. Beck, R.K. Jaiswal, R. Douglas, J.D. Mosca, M.A. Moorman, D.W. Simonetti, S. Craig, D.R. Marshak (1999) Multilineage potential of adult human mesenchymal stem cells. Science *284*: 143-147.

Shimakura, Y., Y. Yamzaki, E. Uchinuma (2003) Experimental study on bone formation potential of cryopreserved human bone marrow mesenchymal cell/hydroxyapatite complex in the presence of recombinant human bone morphogenetic protein-2. J Craniofac Surg *14*: 108-116.

Wang, Z., J. Goh, S. Das De, Z. Ge, H. Ouyang, J.S. Chong, S.L. Low, E.H. Lee (2006) Efficacy of

bone marrow-derived stem cells in strengthening osteoporotic bone in a rabbit model. Tissue Eng *12*: 1753-1761.

Weng, Y., M. Wang, W. Liu, X. Hu, G. Chai, Q. Yan, L. Zhu, L. Cui, Y. Cao (2006) Repair of experimental alveolar bone defects by tissue-engineered bone. Tissue Eng *12*: 1503-1513.

Xiang, Y., Q. Zheng, B. Jia, G. Huang, C. Xie, J. Pan, J. Wang (2007) Ex vivo expansion, adipogenesis and neurogenesis of cryopreserved human bone marrow mesenchymal stem cells. Cell Biol Int *31*: 444-450.

Yamada, Y., M. Ueda, T. Naiki, T. Nagasaka (2004) Tissue-engineered injectable bone regeneration for osseointegrated dental implants. Clin Oral Implants Res *15*: 589-597.

Yoshikawa, T., H. Ohgushi, S. Tamai (1996) Immediate bone forming capability of prefabricated osteogenic hydroxyapatite. J Biomed Mater Res *32*: 481-492.

Zhao, M., Q. Jin, J.E. Berry, F.H. Nociti, Jr., W.V. Giannobile, M.J. Somerman (2004) Cementoblast delivery for periodontal tissue engineering. J Periodontol *75*: 154-161.